An Immunological Study of Pelecypod Taxonomy

BY

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(3 Text figures)

INTRODUCTION

Animal relationships can be studied in many different ways. Organisms are usually classified on the basis of similarities and differences in morphology, in physiology, in development, and in ancestry as shown by fossil remains. Another way that could be used to study relationship would be to consider the amount of difference in the types of substances present in the tissues, such as the proteins present in the muscles. This method was used in this experiment, in which muscle tissue from several species of clams was compared by immunological means.

Immunological methods have been used in the past to study taxonomy of vertebrates. The first extensive work of this sort was done by Nuttall (1904) who showed that when blood sera from many species, including man, the great apes and the domestic cat and dog were used as antigens and tested with rabbit antisera, the reactions were greatest when the species from which the blood serum was taken was more closely related to the species from which the original antigen had been obtained for injection into the rabbit. In Nuttall's study, increasing strength of the antibody-antigen reaction was found to follow a closer zoological relationship, and thus agree with standard taxonomy.

More recently, serological methods have been used in both plant and animal taxonomy, as in the work of Fairbrothers & Johnson (1962) with systemics of the families Cornaceae (Dogwood) and Nyssaceae (Sour Gum); Goodman's (1962) immunological study of primate taxonomy; the serological comparison of 7 species of birds by Duwe (1962); the gel diffusion tests of ungulate taxonomy by Marable & Glenn (1962); and the investigation of turtle family relationships by Frair (1962).

The use of immunological techniques to study taxonomic relationships is based on the assumption that just as closely related clam species have more similarities of gross morphology than do less closely related species, so do they also have more similarities in their tissue proteins. If a tissue, such as muscle, is ground up to break the cell walls and free the proteins, it can then be used as an antigen, that is, a substance to which antibodies can be produced. When this antigen is injected into a rabbit, the rabbit will make antibodies to the foreign proteins and these antibodies will appear in its blood serum. If this antiserum is mixed with the original antigen, the antibody and antigen molecules will attach to one another in great numbers and form a lattice, which is more dense than the surrounding fluid and will form a precipitate. This reaction between the antibodies and the proteins which make up their homologous antigen is quite specific [2]. Thus, if there was a precipitate between antigen from the muscle of one clam and antiserum made against the muscle tissue of another clam, it would show that the two had at least one protein in common. If two such lines of precipitate met and merged, it would show that they were reactions to a single protein, present in both subjects. If they did not merge, however, this would demonstrate that while muscle tissue from each species did have a protein in common with the original antigen to which the antiserum was made, it was not the same protein; therefore each of these two species of clams had one protein in its muscle that the other one did not have, thereby indicating a more distant taxonomic relationship. These were the principles that were used in the present experiment, to examine the amount of relationship shown among several different species of clams.

METHODS

Seven species of clams were used in this experiment, 6 of which occur in the fresh water streams of Illinois, and one that is marine, from the coast of the State of Washington. The fresh water species were:

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[2] see Boyd, 1966, pp. 370 - 371

SPHAERIIDAE
Sphaerium striatinum (LAMARCK, 1818)
UNIONIDAE
Unioninae
Quadrula pustulosa (LEA, 1834)
Anodontinae
Anodonta grandis SAY, 1829
Lasmigona complanata (BARNES, 1823)
Lampsilinae
Lampsilis ventricosa (BARNES, 1823)
Actinonais carinata (BARNES, 1823)
The marine clam was:
VENERIDAE
Saxidomus nuttalli CONRAD, 1837.

The antigen to be injected was obtained from 3 species, *Lampsilis ventricosa*, *Quadrula pustulosa*, and *Lasmigona complanata*. The retractor and adductor muscles were removed, cut into fine pieces, extracted in saline solution, frozen and thawed several times and the same samples were then ground in a hand grinder. The protein concentrations of the antigen preparations were determined by the biuret method, using a colorimeter. Each antigen was injected several times (see antigen schedule below) into 2 rabbits. Injections were made intramuscularly, with the dose divided into 4 injections, one in the proximal part of each limb. At the time of the injections, 15 to 20 cc were bled from an ear of each rabbit, the clot removed and the serum centrifuged to prevent hemolysis. Pre-immunization (control) bleedings were taken from each rabbit before the first injection. The schedule of bleedings, antigen injections and their concentrations is given below.

ANTIGEN SCHEDULE

Antigens from *Quadrula pustulosa*:

1. Antigen concentration – 1.68 mg/ml protein
   Injected – day one: 10 ml antigen for 16.8 mg protein and 5ml Freund's complete adjuvant, thoroughly mixed, into rabbits one and two.
2. Antigen concentration – 0.67 mg/ml protein
   Injected – day nine: 7.5 ml antigen for 5 mg protein and 5 ml Freund's complete adjuvant into the same 2 rabbits.
3. Antigen concentration – 0.60 mg/ml protein
   Injected – day eighteen: 14 ml antigen for 8.4 mg protein and 6 ml Freund's complete adjuvant into rabbit number one only.

Antigens from *Lasmigona complanata*:

1. Antigen concentration – 1.0 mg/ml protein
   Injected – day three: 10 ml antigen for 10 mg protein and 10 ml Freund's complete adjuvant into rabbits three and four.

This was the only injection of *Lasmigona complanata* material as no more specimens of this species could be obtained.

Antigens from *Lampsilis ventricosa*:

1. Antigen concentration – 1.68 mg/ml protein
   Injected – day one: 11.5 ml for 18.3 mg protein and 5.5 ml Freund's complete adjuvant into rabbits five and six.
2. Antigen concentration – 0.4 mg/ml protein
   Injected – day nine: 7 ml for 2.8 mg protein each into the same two rabbits.

The rabbits were bled on days one, nine, eighteen, and thirty-three even when they were not injected.

Diffusion plates were prepared by pouring a 1% solution of agar agar no. 3 (Oxoid Co.) onto 16 plates, and cutting wells $\frac{1}{4}$ inch in diameter and $\frac{3}{8}$ inch apart in substrate $\frac{1}{8}$ inch deep, following the Ouchterlony procedure. Antigens and antisera were placed undiluted in the wells of the plates shown in the diagrams below. The results with the first group of plates (Text figure 1) indicated that dilution of the antisera would improve the readings. Therefore each antiserum was diluted 1:1; 1:4; 1:8; 1:16; 1:32; and 1:64 and tested against its homologous antigen, except for the antisera to *Lasmigona complanata* as no more antigen from this species was available. From the results of these tests, the antisera were diluted 1:6 in the second group of plates, except that from rabbit number six, which was diluted 1:1. All of the plates were observed after 1, 3 and 5 days of refrigeration and the results were recorded.

RESULTS

The patterns of precipitation on the plates are shown on the diagrams below. These are the patterns on the fifth day, when the plates seem to have attained their full development.

Preimmunization (control) bleedings: the absence of precipitation indicated that the rabbits had no antibodies to the clam proteins at the beginning of the experiment.

DISCUSSION AND CONCLUSIONS

The results indicated that each of the clam species used in this experiment was more closely related to some of the remaining 6 than to others. These relationships agreed in the main with conventional taxonomy but showed that more distantly related groups may have a good deal more in common than one might expect. The amount the spe-

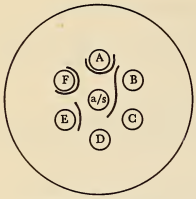

Plate 1
a/s to *Quadrula pustulosa*
from rabbit number one

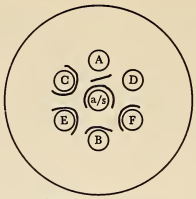

Plate 2
a/s to *Quadrula pustulosa*
from rabbit number two

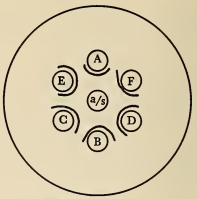

Plate 3
a/s to *Lampsilis ventricosa*
from rabbit number five

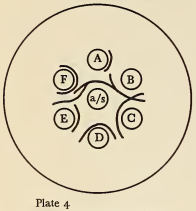

Plate 4
a/s to *Lampsilis ventricosa*
from rabbit number six

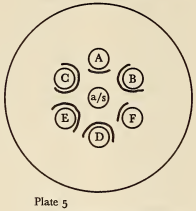

Plate 5
a/s to *Lasmigona complanata*
from rabbit number four

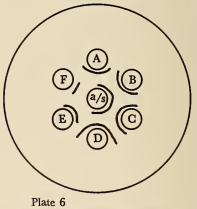

Plate 6
a/s to *Lasmigona complanata*
from rabbit number three

Figure 1

cies share is amplified by the fact that these animals are not closely related to rabbits. According to LANDSTEINER (1936) antibody formation is directed against those features that are foreign to the animal forming the antibody; thus much of a rabbit's antibody forming capacity would be directed against the major structural features not occurring in rabbits, but common to all clam proteins. This would also mean that only part of the antibodies would distinguish the relatively minor species differences among the clam proteins. The diagram (Figure 3) shows the similarities of muscle proteins that were found in this experiment. Each line represents one positive reaction, and if 2 or more antibody-antigen systems precipitated together, it would represent more than one substance in common in their muscle tissues. It must be noted, however, that the gel diffusion tests would not have detected differences or similarities among clam proteins that are also present in rabbits, as the rabbits would not have recognized them as foreign and so would not have produced antibodies against them.

Each antigen could have shown reaction 5 or 6 times, had all reactions been positive. The number of possible positive reactions for each species, the number of those that were prevented because of precipitation too close to the well of antigen and the number of reactions that were actually positive, are shown in Table 1.

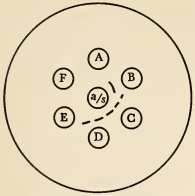

Plate 1
a/s to *Lampsilis ventricosa*
from rabbit number six

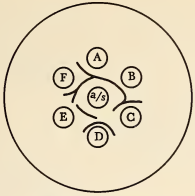

Plate 2
a/s to *Lampsilis ventricosa*
from rabbit number six

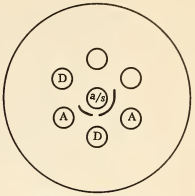

Plate 3
a/s to *Lampsilis ventricosa*
from rabbit number six

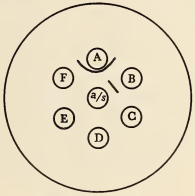

Plate 4
a/s to *Lasmigona complanata*
from rabbit number four

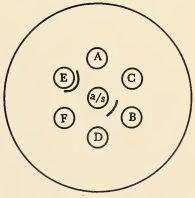

Plate 5
a/s to *Lampsilis ventricosa*
from rabbit number five

Figure 2

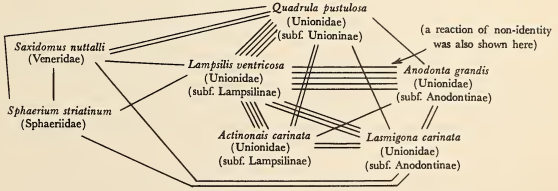

Figure 3
Similarities Shown Among Muscle Proteins

Table 1

Percentage of Positive Tests

Species	Percentage Positive	Possibilities in Experiment	Number Prevented	Valid Possibilities	Positive Tests
Lampsilis ventricosa	100%	6	0	6	6
Lasmigona complanata	100%	6	0	6	6
Quadrula pustulosa	100%	6	0	6	6
Actinonais carinata	80%	6	1	5	4
Saxidomus nuttalli	80%	6	1	5	4
Anodonta grandis	67%	6	0	6	4
Sphaerium striatinum	67%	6	0	6	4

The species at the top of this table showed a positive reaction at least once with each species tested against them, while those in the lower part of the table showed less and less relationship with the other species.

The results of this experiment might have shown more if several other species of bivalves could also have been used, and if the antisera had been diluted for the first group before being placed in the wells so that precipitation would have taken place farther from the wells of antigen. However, the results showed two things: first, that groups considered more distantly related do have a good deal of similarity in their muscle proteins, and, secondly, that classification by common factors in muscle proteins agrees substantially with the classification based on morphology, physiology and development.

SUMMARY

The amount of relationship among 7 species of bivalves was compared by immunological methods, using their muscle tissue as the antigen and testing for similarity of muscle proteins of the different species using the Ouchterlony technique. Six of the species were from fresh water, representing 2 families and within one of these 3 subfamilies. The seventh species was marine from the Pacific coast. The results showed 2 things: bivalves more distantly related to one another have a good deal of similarity in their muscle proteins, and that classification of pelecypods by common factors in muscle proteins agrees substantially with that based on morphology, physiology and development.

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